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Bioreactor culture of oil palm (*Elaeis guineensis*) and effects of nitrogen source, inoculum size, and conditioned medium on biomass production

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Abstract

We report the successful culture of oil palm (*Elaeis guineensis* Jacq.) suspension cells in a bioreactor. In vitro propagation of this perennial monocotyledonous tree is an important part of the oil palm industry's approach to clonal propagation of high-yielding accessions. During culture of oil palm cells in a batch bioreactor, nutrients and extracellular metabolites were monitored, and kinetic parameters and nutrient-to-biomass conversion yields were calculated. The biomass increased approximately 3.5-fold per month, consistent with values reported for shake flask cultures. Although the carbon source was completely depleted by the end of the run, nitrogen sources remained in large excess and the sugar-to-biomass conversion yield remained low. Linear growth indicated that the cells were limited. The results obtained from the bioreactor runs indicated that we should be able to improve biomass production by carrying out optimization studies. Therefore, we initiated multi-factorial analyses using response surface experimental designs to investigate the effects of different nitrogen sources, as well as inoculum size and conditioned medium, on biomass production in flask cultures. Whereas glutamine does not have a significant effect on biomass production, ammonia has a positive effect up to an optimum concentration. Both inoculum density and conditioned medium have positive, synergistic effects on biomass production.
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Keywords: Bioreactor; Inoculum size; Conditioned medium; Response surface methodology; Elemental composition; Palm oil

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1. Introduction

Oil palm (*Elaeis guineensis* Jacq.), a perennial allogamous monocotyledonous tree, gives the highest yield of oil per hectare of any oil-producing crop. Palm oil, palm kernel oil and specific oils derived therefrom are used in the production of cooking oils, margarines, soap, and detergents. Given the increasing economic value of oil palm, micropropagation of this tropical crop has been studied to develop oil palm propagation with a view to the commercial distribution of elite clones capable of increased oil production (de Touchet et al., 1991).

Clonal propagation of oil palm has been studied for many years as a potential way to develop high-yielding collections while circumventing the long generation time required with traditional breeding techniques. In 1991, the first regenerable oil palm embryogenic cell suspension cultures were obtained from oil palm leaf-derived calli (de Touchet et al., 1991). Later, mature zygotic embryos excised from fruits were used to generate friable embryogenic tissue and to establish embryogenic suspensions (Teixeira et al., 1995). Although these two protocols were successful for establishing single somatic embryos from embryogenic suspension cultures, the regeneration rate into plantlets was poor. This issue was overcome by Aberlenc-Bertossi et al. (1999), who developed a protocol to improve the quality of shoot formation. The first results obtained from suspension culture-derived clones are encouraging and field trials are under way (Rival et al., 2001).

Cell suspension cultures have been extensively utilized in oil palm micropropagation. However, no references are available regarding the use of bioreactors for micropropagation of oil palm. Bioreactors have been successfully used for somatic embryo production with at least fourteen plant species (reviewed in Ibaraki and Kurata, 2001). One advantage of using bioreactors in culture processes is that culture parameters, such as pH, temperature, dissolved oxygen concentration and agitation can be monitored and controlled. Therefore, it is possible to study cell behaviour by determining growth kinetics, nutrient uptake and metabolite production. Such information is essential to identify the key factors for optimization studies.

Parameters such as nitrogen source, use of conditioned medium, and inoculum size have been shown to influence the quality of cell suspension cultures in a species-dependent manner. The significant role of the nitrogen source has been well demonstrated in different cell suspension cultures such as *Catharanthus roseus* (Rho and André, 1991), rice (Hayashi et al., 1994), maize (Padgett and Leonard, 1996), *Panax ginseng* (Liu and Zhong, 1997), and *Hordeum vulgare* L. (barley) (Nuutila et al., 2000). However, no information is available on the effects of nitrogen sources in oil palm cell suspension cultures. Inoculum size and the presence of conditioned medium have been independently shown to play a role in oil palm cell suspension culture (de Touchet et al., 1991; Teixeira et al., 1995). However, the possible interaction between those two factors has never been investigated.

It is likely that factors influencing biomass production of plant cells are interdependent. Therefore, a multifactorial approach appears to be more appropriate than the approach of varying one factor at a time (Payne et al., 1991). Multifactorial approaches have been successfully applied in other plant cell systems (Weathers et al., 1997; Nuutila et al., 2000).

In order to improve the process of oil palm micropropagation and to gain a better understanding of the mechanisms involved in oil palm somatic embryogenesis, we initiated investigations on suspension cultures in bioreactors. Here, we describe for the first time the culture of oil palm in a bioreactor. Kinetic data were obtained to provide a better understanding of the behaviour of the cell. Data obtained from the growth of oil palm cells in the bioreactor suggested the importance of key parameters, and preliminary optimization studies were carried out using response surface experimental designs.

2. Materials and methods

2.1. Oil palm suspension flask culture

Oil palm cell suspension cultures were furnished by the Malaysian Palm Oil Board (MPOB) (Kuala Lumpur, Malaysia). The cell suspension cultures were produced from leaf meristem-derived calli. The oil palm cells were subcultured every two weeks. The cells were passed through a sterile brass sieve and transferred to a new flask containing Murashige and Skoog (MS) medium (Gibco, Grand Island, NY,

USA) that was supplemented with 1 mg l^{-1} 2,4-D (dichlorophenoxy-acetic acid), 1 mg l^{-1} NAA (naphthaleneacetic acid) and 100 mg l^{-1} glutamine. The cells were incubated at 26 °C and agitated at 125 rpm.

2.2. Oil palm bioreactor

The fermentations were carried out in Sixfors bioreactors (Infors, AG CH-4103 Bottmingen/Switzerland). The glass vessels were custom modified at the bottom by the addition of a large bore port with a 15° angle slant oriented toward the top to allow accurate sampling for biomass measurement. The working volume was 300 ml. Four to six grams of fresh weight (Fw) of cells were used to inoculate 300 ml of MS medium supplemented as described. Temperature, aeration and agitation were regulated at 26 °C, 20% dissolved oxygen (DO) of saturated air and 150 rpm, respectively. The DO was controlled with a discontinuous air flow entrance regulated by a solenoid valve. At the beginning of the run, the 20% DO was maintained by addition of nitrogen gas. The bioreactors were equipped with two 2.5 cm diameter marine-type impellers. The pH was not controlled. Cultures were carried out in duplicate.

2.3. Determination of dry weight

Twelve milliliter samples were collected regularly during the run and centrifuged at 3500 × *g* for 10 min. The supernatant was filtered through 0.2 μm Acrodisc filters (Pall Gelman, Ann Arbor, MI, USA) and stored at −20 °C for further analysis. The cells were washed twice with cold distilled water and dried using a vacuum oven at 80 °C for 48 h. The dry weight (Dw) was quantified gravimetrically.

2.4. Determining molecular composition of oil palm cells

A 50 ml culture sample from a two week old flask culture was centrifuged at 12,000 g for 5 min. The cells were washed twice with cold distilled water and dried using a vacuum oven at 80 °C for 48 h. Duplicate samples of dried oil palm cells, as well as duplicate samples of *Escherichia coli* as a control, were sent to EAI (Elemental Analysis-Inc, KY, USA) for C, H, O, N determination.

2.5. Determination of sugars, organic acids, ammonia, nitrates, amino acids and total phenolics

Twelve milliliter samples were collected regularly during the run and centrifuged at 3500 × *g* for 10 min. The supernatants were filtered through 0.2 μm Acrodisc filters. Sugar and organic acid concentrations were determined by HPLC (Hewlett Packard model 1050, Waldbronn, Germany) using an Aminex® HPX-87H column (Bio-Rad, Hercules, CA, USA). Sample analysis was performed at 40 °C using 5 mM sulfuric acid as the mobile phase at a flow rate of 0.6 ml min^{-1}. The detection of sugars was performed with a refractive index detector (Hewlett Packard model 1047A) and the detection of organic acids with the UV detector at 210 nm (Hewlett Packard model 1050). Amino acids were analyzed as ortho-phthaldialdehyde (OPA) derivatives by reversed−phase chromatography using a C18 AminoQuant column with a Hewlett Packard series 1050 high-pressure liquid chromatography (HPLC) system (Hewlett Packard). Nitrate and nitrite were analyzed using a colorimetric method (Cat No. 1 746 081) (Roche, Mannheim, Germany). Ammonia was analyzed using an enzymatic method (Cat No. 171-UV) (Sigma, St. Louis, MO, USA). Total phenolics were analyzed using a modified Folin-Ciocalteu colorimetric method (Gao et al., 2000).

2.6. Determination of glutamine chemical breakdown

Five flasks containing the same medium used for suspension culture were kept in the incubator at 26 °C, 125 rpm for 12 days in the absence of cells. A sample from each flask was taken on a daily basis and analyzed for glutamine and glutamate using the amino acid HPLC method described above.

2.7. Experimental design: NH_4NO_3–glutamine

2.7.1. Design

A response surface experimental design using a quadratic model was built with StatGraphicsPlus 5.1 software (Manugistics, Rockville, MD, USA). Two factors were investigated: NH_4NO_3 concentration (0–1.76 g l^{-1}), and glutamine concentration (0–206 mg l^{-1}). Each factor had five levels (Table 1).

Table 1
Levels of variables used in the $(2^2 + 5)$ central composite experimental design used for studying the effects of ammonia and glutamine supplemented in the medium on biomass production of oil palm cells (expressed as fresh weight (Fw))

Coded values	NH_4NO_3 concentrations ($g\,l^{-1}$)	Glutamine concentration ($g\,l^{-1}$)
$-\infty$	0.000	0
-1	0.250	25
0	0.875	100
$+1$	1.500	175
$+\infty$	1.759	206

The design contained 13 experiments, 8 unique combinations and 5 replications at the central point (NH_4NO_3 0.875 $g\,l^{-1}$, glutamine 100 $mg\,l^{-1}$). Replication of the central point five times provided sufficient degrees of freedom for estimating the purely experimental error. Fresh weight (Fw) was used as the response. A second order polynomial model was defined to fit the responses.

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j$$

where Y is response, β_0 is the constant coefficient, X_i are coded variables, β_i are linear coefficients, β_{ij} are the second order interaction coefficients and β_{ii} are the quadratic coefficients.

The correspondence between the natural and coded values can be obtained by:

$$X_i = \frac{x_i - x_0}{\delta x}$$

where X_i is the coded value, x_i is the corresponding natural value, x_0 is the natural value in the center of the domain, and δx is the increment of x_i corresponding to 1 unit of X_i.

2.7.2. Experiments

The experiments were carried out in 125 ml flasks. The medium used was an MS based medium modified as described in Table 2. The hormones 2,4-D and NAA were added as described above. The cells were incubated at 26 °C at 125 rpm. After 30 days, the cells were harvested and the total biomass expressed in fresh weight (Fw) was used as a response for technical reasons. In order to express the result as "total biomass produced" it was necessary to subtract the inoculum weight from the final biomass weight. Therefore, the final weight was measured using the same non-destructive fresh weight method that was used to measure the inoculum weight.

Table 2
Experimental results of the $(2^2 + 5)$ central composite experimental design used to study the effect of ammonia and glutamine supplemented in the medium on biomass production of oil palm cells (expressed as fresh weight (Fw))

Experimental design	Variables		Response
	NH_4NO_3	Gln	Biomass (g Fw)
	Coded value[a]	Coded value[a]	
1	-1	1	1.5705
2	$-\infty$	0	1.1757
3	0	$+\infty$	1.7843
4	0	$-\infty$	1.6359
5	**0**	**0**	**1.8980**
6	**0**	**0**	**1.7779**
7	**0**	**0**	**1.7982**
8	1	1	2.0105
9	**0**	**0**	**1.8340**
10	**0**	**0**	**1.8322**
11	1	-1	1.9050
12	-1	-1	1.4211
13	$+\infty$	0	1.8462

Bold values correspond to the central point repeated five times.
[a] See Table 1 for definition of coded values.

2.8. Experimental design: inoculum size and conditioned medium

2.8.1. Design

Two factors were investigated: inoculum size (0.07–0.92 g Fw l^{-1}) and conditioned medium (0–10.65 ml). Each factor had five levels (Table 3). The design contained 13 experiments as with the above experiment.

2.8.2. Experiments

The experiments were carried out in 125 ml flasks. The medium used was an MS-based medium modified as described in Table 4. "Conditioned medium" is the culture supernatant from 2-week-old oil palm cultures. The volume of the medium was kept constant at 30 ml. The hormones 2,4-D and NAA were added as described above. The cells were incubated

Table 3
Levels of variables used in the $(2^2 + 5)$ central composite experimental design used for studying the effects of inoculum size and conditioned medium on biomass production (expressed as fresh weight (Fw))

Coded value	Inoculum size (g)	Conditioned medium (ml)
$-\infty$	0.076	0.00
-1	0.200	1.00
0	0.500	5.00
$+1$	0.800	9.00
$+\infty$	0.924	10.65

Table 4
Experimental results of the (2^2+5) central composite experimental design used to study the effect of inoculum size and conditioned medium on biomass production (expressed as fresh weight (Fw))

Experiments	Variables		Response
	Inoculum size	Conditioned medium	Biomass (g Fw)
	Coded value[a]	Coded value[a]	
1	$+1$	$+1$	1.8683
2	-1	-1	0.7843
3	$-\infty$	0	0.8855
4	$+1$	-1	2.1454
5	**0**	**0**	**2.0238**
6	**0**	**0**	**2.1861**
7	**0**	**0**	**2.0468**
8	0	$-\infty$	1.3917
9	$+\infty$	0	2.1503
10	**0**	**0**	**2.2312**
11	0	$+\infty$	1.9618
12	**0**	**0**	**1.9251**
13	-1	$+1$	1.2125

Bold values correspond to the central point repeated five times.
[a] See Table 3 for definition of coded values.

at 26 °C and agitated at 125 rpm. After 30 days, the cells were harvested and the total biomass expressed in fresh weight (Fw) was used as a response for the same technical reasons described above.

3. Results

3.1. Oil palm bioreactor

Cultures of oil palm cells were successfully grown in batch bioreactors. From these, kinetic data on growth, nutrient uptake and metabolite production were determined (Fig. 1). The biomass multiplied 3.5-fold in about 25 days. The biomass was composed of a mixture of fine cells and aggregates with sizes between 0.1 and 0.25 mm. The growth kinetic data show that the cells entered a linear growth phase after 10 days with a growth rate of 0.13 g Dw l^{-1} per day (Table 5). After 22 days, a decrease in dry weight was observed.

Table 5
Kinetics and yield data from a typical culture of oil palm cells in a batch bioreactor

Growth		
Total growth	3.1	g Dw l^{-1}
Linear growth rate	0.13	g Dw l^{-1} per day
Maximum specific rates		
$q_{\text{sugarsMax}}$	0.45	gC g^{-1} Dw per day
$q_{\text{NitrateMax}}$	13	mg nitrate g^{-1} Dw per day
$q_{\text{AmmoniaMax}}$	1.8	mg ammonia g^{-1} Dw per day
$q_{\text{GlutamineMax}}$	11.51	mg glutamine g^{-1} Dw per day
Yields		
$Y_{X/\text{sugar}}$	0.18	g Dw g^{-1} sugar
$Y_{X/\text{Nitrogen}}$	0.24	g Dw mM^{-1} nitrogen

The pH was monitored during the course of the culture. The pH decreased rapidly from 5.6 to 4.0 during the first 9 days and then increased gradually to reach pH 4.4 after 25 days. At this point in the run the cells transitioned from growth to stationary phase and a dramatic increase to pH 5.5 was observed. The carbon source, sucrose, was first hydrolysed to fructose and glucose. The monosaccharides were then consumed simultaneously. The carbon source was completely depleted by the end of the run (Fig. 1).

Three nitrogen sources are present in the medium: two inorganic forms (ammonia and nitrogen) and one organic form (glutamine). Only 30% of the inorganic nitrogen was consumed over the course of the culture. The uptake of these two nitrogen sources started only after 5 days. During the growth phase, ammonia and nitrate were taken up at the same time. After 25 days, the uptake of ammonia stopped while nitrate was still being used. The nitrate kinetic appears to begin with an uptake phase, followed by an excretion phase, then by a second uptake phase. The organic nitrogen source, glutamine, was completely depleted after 5 days. To address the possibility that the glutamine was breaking down chemically, we carried out a kinetic analysis of the chemical decomposition of glutamine under our

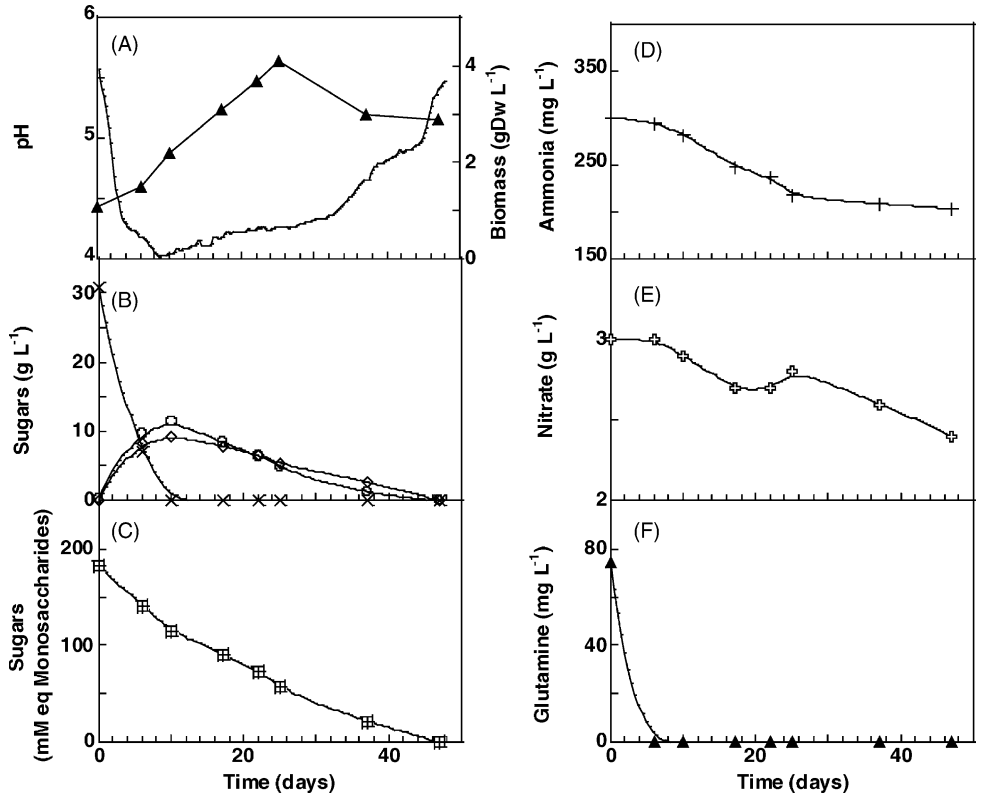

Fig. 1. Changes in pH, biomass and nutrient uptake during suspension culture of oil palm cells in a batch bioreactor: (A) pH (—), biomass (▲); (B) sucrose (×), glucose (◇); and fructose (○); (C) total sugars data from panel B expressed as mM equivalents of monosaccharide; (D) ammonia (+); (E) nitrate (✠); (F) glutamine (▲).

culture conditions in the absence of cells. This analysis demonstrated that glutamine did not break down during the first 6 days under these conditions (data not shown).

Extracellular metabolites were also measured (Fig. 2). Amino acids were excreted into the medium at the end of the run after growth of the cells had stopped. Alanine, homoserine and isoleucine were the main amino acids present in the medium. We measured phenolic compounds and showed that phenolic compounds or interfering reducing substances were excreted at only a low level during the run. No organic acids were produced at a significant level over the course of the fermentation.

3.2. Mass balances

The final balances of carbon and nitrogen sources were assessed using the oil palm elemental formula $CH_{1.76}O_{0.56}N_{0.10}$ calculated from the values obtained for the C, H, O, N composition. For the carbon balance, the consumed sugars and glutamine accounted for 20% of biomass and 1% of metabolites (mainly amino acids). The final nitrogen balance was closed at 95%. Eighty percent of ammonia, nitrate and glutamine went into biomass and 15% into amino acids.

3.3. Kinetic parameters and production yields

The kinetic parameters and the production yield were calculated from the kinetic data and are summarized in Table 5. The maximal specific uptake rates were obtained at the beginning of growth during the first 10 days. The production yields $Y_{Biom/Sugar}$ and $Y_{Biom/Nitrogen}$ were constant during the first 25 days with values of $0.18\,g\,Dw\,g^{-1}$ sugars and $0.24\,g\,Dw\,mM^{-1}$ nitrogen, respectively.

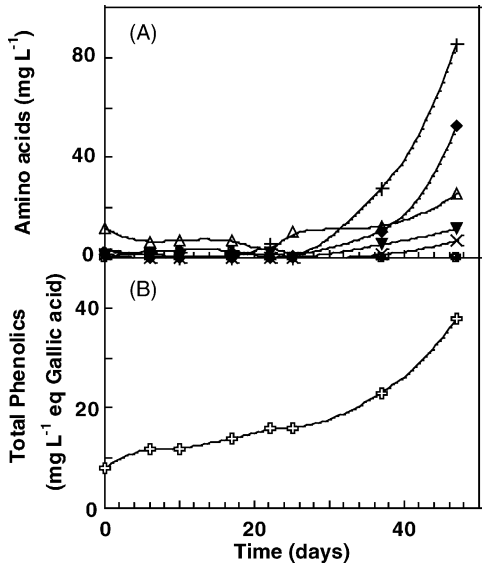

Fig. 2. Changes in extracellular metabolite accumulation during suspension culture of oil palm cells in a batch bioreactor: (A) extracellular amino acids: aspartate (●), glutamate (○), homoserine (◆), threonine (×), alanine (+), isoleucine (△), leucine (▼); (B) total phenolics (✠).

Table 6
R-squared of the polynomial model and the *P*-values of linear, quadratic and interaction effects of ammonia and glutamine supplemented in the medium on biomass production of oil palm cells expressed as fresh weight (Fw)

	Biomass (g Fw)
R-squared	94%
R-squared adjusted	90%
P-value	
Linear	
NH_4NO_3	0.0000
Gln	0.0549
Quadratic	
$NH_4NO_3 \times NH_4NO_3$	0.0020
Gln × Gln	0.3056
Interaction	
$NH_4NO_3 \times$ Gln	0.7680

3.4. Experimental design

3.4.1. NH_4NO_3 and glutamine

To test the role of the glutamine and other nitrogen sources on biomass production we carried out a multifactorial experimental design. Experimental data for each condition are summarized in Table 2. Growth was possible in the entire tested domain and varied from 1.1 to 2.0 g total fresh weight (Fw).

The statistical analysis of the results was performed using StagraphicsPlus 5.0 software. The *P*-value of the effect of each factor and their interaction was obtained by an ANOVA test with a 95% level of confidence. The results of the variance analysis are reported in Table 6 and indicate that the model developed for biomass has both a good *R*-square (94%) and *R*-square adjusted (90%), meaning that 90% of the variation is explained by the regression model. Diagnostic plots were analyzed to assess the strength of the model. The error distribution was scattered with no observable pattern and the residual distribution was normal. As indicated by the *P*-value, NH_4NO_3 has a positive effect until it reaches an optimum (data not shown). For glutamine, the linear and the quadratic effect are not significant at 95% ($P > 0.05$). There is no significant interaction between ammonia and glutamine.

In order to determine the optimum value, the model was simplified by removing the non-significant interaction effects. Three different conditions were tested to check the optimum condition predicted by the model: the optimal condition given by the model (1.45 g l^{-1} NH_4NO_3 and 206 mg l^{-1} glutamine), Test (1) without any glutamine (1.45 g l^{-1} NH_4NO_3 and no glutamine) and Test (2) with the previously reported concentration of glutamine (1.45 g l^{-1} NH_4NO_3 and 100 mg l^{-1} glutamine) (Table 7). Four replicates were carried out for each of the conditions. A *t*-test was carried out to test the difference between each condition and showed that with or without glutamine the biomass production was similar.

3.4.2. Inoculum density and conditioned medium

Previous reports showed that inoculum density and conditioned medium can influence biomass production in oil palm cells and we investigated their effect and their possible interaction using experimental design. Experimental data for each of the conditions are summarized in Table 4. Growth was possible in the entire tested domain and varied from 0.8 to 2.2 g total fresh weight (Fw). Data were treated as previously described. The results of the variance analysis are reported in Table 8 and indicate that the model developed

Table 7
Flask experiments carried out to check the accuracy of the optimum defined by the regression model

Experiments		Predicted value (biomass in g Fw)	Experimental data (biomass in g Fw)
1.45 g l^{-1} NH_4NO_3 206 mg l^{-1} glutamine	Optimum condition	1.95	2.09 ± 0.15
1.45 g l^{-1} NH_4NO_3 no glutamine	Test 1		2.31 ± 0.16
1.45 g l^{-1} NH_4NO_3 100 mg l^{-1} glutamine	Test 2		2.16 ± 0.09

for biomass has both a relatively good *R*-square (95%) and *R*-square adjusted (92%), meaning that 92% of the variation is explained by the regression model. Diagnostic plots were analysed to assess the strength of the model. The error distribution was scattered with no observable pattern and the residual distribution was normal. As indicated by the *P*-values, inoculum density has significant linear and quadratic effects ($P < 0.05$). Conditioned medium has also significant linear and quadratic effects but at a lower level. Both inoculum density and conditioned medium have a positive effect until they reach an optimal value. Interaction between inoculum density and the amount of conditioned medium are significant.

The optimal condition was estimated using the complete model. Indeed, each of the factors were significant and could not be removed from the model. The optimum conditions given by the model were 0.7 g of inoculum and 5 ml of conditioned medium, which correspond to the central point for the conditioned medium. Five replicates were carried out to test the accuracy of the estimation. The value obtained was 2.09 ± 0.05 g Fw, similar to the value 2.26 g Fw predicted by the model.

Table 8
R-squared of the polynomial model and the *P*-values of linear, quadratic and interaction effects of inoculum size and conditioned medium on biomass production of oil palm cells (expressed as fresh weight (Fw))

	Biomass (g Fw)
R-squared	95%
R-squared adjusted	92%
P-value	
Linear	
Inoculum size	0.0000
Conditioned medium	0.0482
Quadratic	
Inoculum size × inoculum size	0.0007
Conditioned medium × conditioned medium	0.0039
Interaction	
Inoculum size × conditioned medium	0.0416

4. Discussion

4.1. Oil palm bioreactor

We have been able to demonstrate that embryogenic oil palm cells can grow successfully in a stirred-tank bioreactor with an increase in biomass of ~3.5-fold per month. This result agrees with the data reported by de Touchet et al. (1991) for oil palm flask cultures, as well as for other monocotyledons such as wheat (Redway et al., 1990), maize (Emons and Kieft, 1995) and banana (Cote et al., 1996).

The appearance of the linear phase shows that the culture was limited. In plant cell cultures, nitrogen sources (Rho and André, 1991; Wroblewski et al., 1995; Liu and Zhong, 1997), phosphate and trace ions sources (Curtis et al., 1991), conditioned medium (Lee and Shuler, 2000; Woragidbumrung et al., 2001), inoculum density (Lee and Shuler, 2000) and CO_2 accumulation (Mirjalili and Linden, 1995; Hohe et al., 1999) are known to be the main limiting factors. The decline phase observed after depletion of carbon source has been noticed for other plant cells (Rho and André, 1991; Tautorus et al., 1992; Liu and Zhong, 1997; Woragidbumrung et al., 2001). Rho and André (1991) explained this decrease in biomass as an endogenous respiration of reserve materials previously stored within the cells. Free amino acids that we observed in the medium during the decline phase reinforce this hypothesis. Indeed, one can imagine that the depletion of the carbon source could induce the proteolysis of storage protein for the carbon supply.

The pH profile observed is characteristic of plant cell cultures carried out without pH control (Hahn et al., 1997; Lee and Shuler, 2000). The initial decrease in pH has been reported many times to be partially associated with ammonium uptake (McDonald and Jackman, 1989; Do and Cormier, 1991; Hahn et al., 1997; Lee and Shuler, 2000). At the end of growth, one can observe a drastic increase of pH. Tautorus et al. (1992) claimed that pH value may be useful for determining specific growth stages, in which pH 6.5–6.8 corresponds to stationary phase in their system.

Kinetic profiles of nutrient uptake and metabolite production are described in this study. Similar profiles of carbon source consumption were described for maize cell suspension cultures (Hahn et al., 1997). Simultaneous uptake of both monosaccharides (glucose and fructose) present in the culture medium precludes the determination of the individual contribution of each sugar. Nevertheless, expressing the carbon source in terms of total monosaccharides allows measurement of the uptake rate (Rho and André, 1991; Liu and Zhong, 1997; Woragidbumrung et al., 2001). The biomass yield coefficient $Y_{X/sugar}$ obtained (0.18 g Dw g^{-1} sugar) was low compared to the values reported in the literature for other plants. For example, values from 0.31 to 0.78 g g^{-1} were reported for *Catharanthus roseus*, and from 0.42 to 0.52 g g^{-1} for *Eschscholtzia californica* (Rho and André, 1991). The values for *Panax ginseng* ranged from 0 to 0.34 g g^{-1} and were shown to depend on the NH_4:NO_3 ratio (Liu and Zhong, 1997). For a ratio similar to that used in MS medium, they reported a biomass yield coefficient value equal to 0.26 g g^{-1}, which is still higher than what we observed. A $Y_{X/sugar}$ closer to 0.50 g g^{-1} would have been a good indication of an active metabolism whereby half of the substrate was converted to biomass or reserve material while the other half was oxidized to produce energy for biosynthetic activities as well as for cell maintenance (Liu and Zhong, 1997). Indeed, the carbon balance shows that only 20% of the carbon used was converted into biomass which mean that the other 80% were converted into non-measured products such as CO_2, ethylene, or intracellular storage substances. Oil palm cells are known to produce phenolics in excess and for this reason activated charcoal is often included in the culture medium for oil palm (de Touchet et al., 1991; Teixeira et al., 1993, 1994, 1995). Although our medium does not contain any activated charcoal the amount of phenolics determined in the medium was low. Storage substances and intracellular composition were not measured in samples from these cultures due to the low biomass activity and the limited volume of the bioreactor. Unfortunately, no data are available on CO_2 or ethylene for oil palm cell suspension cultures and because of the discontinuous air-feed and other equipment constraints we were unable to measure the exhaust gas during the course of the culture in this series of experiments.

Regarding the nitrogen sources, the profiles that we observed for ammonia and nitrate are quite similar to the profiles described in the literature for other plants (Hahn et al., 1997; Liu and Zhong, 1997). A large excess of nitrogen remained in the medium at the end of the run. Usually, with concentrations close to those in MS medium (39 mM for nitrate and 21 mM for ammonia), most of the nitrogen should have been taken up (Hahn et al., 1997; Liu and Zhong, 1997). Nevertheless, the apparent biomass yield obtained was $Y_{X/nitrogen}$ 0.24 g Dw mM^{-1} nitrogen which is similar to the value given by Rho and André (1991). We were able to close the final nitrogen balance at 95%, with the other 5% probably corresponding to storage material. Nevertheless, for most of the time points, instantaneous nitrogen balances could not be closed, which reveals that accumulation likely occurs during the course of the culture. Liu and Zhong (1997) have demonstrated that the intracellular concentrations of nitrate and ammonia varied over the course of the culture of *Panax ginseng*, with accumulation dependent on the NH_4:NO_3 ratio. For instance, they reported that for a ratio similar to that used in MS medium (20:40 mM), the intracellular nitrate varied extensively over the course of the culture.

Glutamine, a possible nitrogen source in our medium, was completely depleted after 5 days. It is well known that the lifetime of glutamine is dependent on the medium (Ozturk and Palsson, 1990) and culture conditions (Bray et al., 1949). For example, glutamine is known to undergo deamination to ammonia during storage (Glacken et al., 1986). Our results showed that in the absence of cells no chemical degradation was observed after 6 days, suggesting that glutamine may have been taken up by the cells. However, labeling experiments would be required to unambiguously demonstrate uptake. Padgett

and Leonard (1996) reported that amino acids are preferentially absorbed in the early stages of culture growth and that active nitrate uptake starts after total depletion of the free amino acids.

To be able to carry out nitrogen and carbon balances, we determined the elemental composition of oil palm cells during the active growth phase. This information is quite important because this type of data is very rare for plant cells. The formula $C_1H_{1.76}O_{0.56}N_{0.10}$ appears to be similar to the formula described for *C. roseus* ($C_1H_{1.57}O_{0.62}N_{0.11}$) determined during the active growth phase (Rho and André, 1991).

The bioreactor experiment pointed out that growth and biomass production are not optimal and probably could be improved. Therefore we initiated some preliminary optimization studies. Mutifactorial experimental designs were carried out to simultaneously study individual and synergistic effects of the chosen factors.

Taking into account the large excess of inorganic nitrogen sources in the medium at the end of the run and knowing the potential toxicity of ammonia for plant cells (Kaul and Hoffman, 1993), we decided to study the effect of NH_4NO_3 on biomass production. Furthermore, after discovering that a rapid decrease in glutamine occurs in the medium, we wished to investigate whether glutamine could be a preferential nitrogen source for the cells. The results from this experimental design show that we should be able to decrease the nitrogen concentration by removing glutamine and reducing the concentration of NH_4NO_3 from 1.60 to $1.45\,g\,l^{-1}$ without affecting the biomass production. The data also suggest that the excess of nitrogen is not responsible for the growth limitation.

Inoculation level and addition of conditioned medium are known to affect the biomass production of plant cells in suspension culture. Our results show that inoculum density and conditioned medium have significant and interdependent effects. The positive effect of the conditioned medium may be due either to a lower osmolarity, due mainly to a decrease in sugar and nitrogen concentrations, or to a change in hormone concentration, or to the presence of stimulating factors. At this level of analysis, we are unable to distinguish between the three possibilities. Lee and Shuler (2000) demonstrated that for *Catharanthus roseus* cells, the positive effect of the conditioned medium is due to both a lower osmolarity and the presence of a component in the cultured medium. The presence of active compounds could explain the interaction between inoculum size and conditioned medium. Earlier studies with oil palm suspension cultures examined the effect of inoculum density on biomass production, although in those studies the amount of conditioned medium was held constant. de Touchet et al. (1991) concluded that the best inoculum density was between 0.1 and 0.3 g per 20 ml of medium that did not include conditioned medium. Teixeira et al. (1995) used medium that contained 25% conditioned medium and found that the best inoculum density was 1.5 g in 50 ml. The optimal condition determined in our multifactorial analysis defined an inoculum density of 0.7 g in 30 ml medium containing 5 ml of conditioned medium.

We report the successful growth of oil palm suspension cultures in a bioreactor. Even under non-optimized conditions, biomass multiplied 3.5-fold in about a month, consistent with previously described results for oil palm flask suspension cultures. Furthermore, for the first time we have also been able to develop a better understanding of oil palm cell suspension with respect to growth, nutrient uptake and metabolite production. Favorable conditions regarding nitrogen sources, inoculum size and conditioned medium have been determined. Other experimental designs are underway to study the effect of medium components (oligoelement, phosphate and hormones) and also to study the effect of process conditions such as aeration and agitation. The gathering of all this information will allow improvements in the culture of oil palm in bioreactors for metabolic studies and micropropagation.

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